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Cloning and Characterization of a Gene Whose Product Is a *trans*-Activator of Anthrax Toxin Synthesis

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The 184-kb *Bacillus anthracis* plasmid pXO1, which is required for virulence, contains three genes encoding the protein components of anthrax toxin, *cya* (edema factor gene), *lef* (lethal factor gene), and *pag* (protective antigen gene). Expression of the three proteins is induced by bicarbonate or serum. Using a *pag-lacZ* transcriptional construct to measure *pag* promoter activity, we cloned in *Bacillus subtilis* a gene (*atxA*) whose product acts in *trans* to stimulate anthrax toxin expression. Deletion analysis located *atxA* on a 2.0-kb fragment between *cya* and *pag*. DNA sequencing identified one open reading frame encoding 476 amino acids with a predicted M_r of 55,673, in good agreement with the value of 53 kDa obtained by in vitro transcription-translation analysis. The cloned *atxA* gene complemented previously characterized Tn*917* insertion mutants UM23 tp29 and UM23 tp32 (J. M. Hornung and C. B. Thorne, Abstr. 91st Gen. Meet. Am. Soc. Microbiol. 1991, abstr. D-121, p. 98), which are deficient in synthesis of all three toxin proteins. These results demonstrate that the *atxA* product activates not only transcription of *pag* but also that of *cya* and *lef*. β-Galactosidase synthesis from the *pag-lacZ* transcriptional fusion construct introduced into an insertion mutant (UM23 tp62) which does not require bicarbonate for toxin synthesis indicated that additional regulatory genes other than *atxA* play a role in the induction of anthrax toxin gene expression by bicarbonate.

Bacillus anthracis causes the highly infectious disease anthrax in animals and humans. Two major virulence factors of *B. anthracis* are known: a poly-D-glutamic acid capsule and a tripartite toxin (20) consisting of protective antigen (PA), edema factor (EF), and lethal factor (LF). Each of these virulence factors is encoded on a distinct, large plasmid (11, 23, 38). The *cap* region, which is essential for encapsulation of *B. anthracis*, is located on a 97-kb plasmid (11, 21, 22, 37, 38), and the three toxin genes, *pag* (PA gene), *cya* (EF gene), and *lef* (LF gene), are located on the 184-kb plasmid pXO1 (26, 29, 34). Each of the anthrax toxin components is inactive when administered alone, but binary combinations cause two distinct toxic effects in experimental animals. Thus, PA together with LF kills certain susceptible animals, whereas intradermal injection of PA with EF causes edema in guinea pigs or rabbits. EF is known to be a calmodulin-dependent adenylate cyclase (18, 19), and evidence was recently presented that LF is a metalloprotease (17). Reviews describing the properties of the toxin (19, 20) and development of improved anthrax vaccines (36) are available.

Although both capsule and toxin are expressed during in vivo infections, their synthesis in vitro is strongly influenced by medium composition (9, 33). Thus, *B. anthracis* produces a polyglutamate capsule during in vitro culture only when serum or bicarbonate is added. (In this report, the term "bicarbonate" will represent the mixture of CO_2, H_2CO_3, HCO_3^{1-}, and CO_3^{2-}, all of which are in equilibrium, with their amounts determined by pH.) Similarly, only small amounts of the anthrax toxin proteins are obtained from common rich media, whereas cells grown in certain synthetic media produce substantial amounts of toxin proteins, but only when these media are supplemented with bicarbonate (19, 41). Therefore, expression of both capsule and toxin appears to be controlled by genetic regulatory mechanisms that sense bicarbonate. Bartkus and Leppla (1) reported that production of PA is transcriptionally regulated by bicarbonate, that a *trans*-acting factor is required for *pag* transcription, and that at least some of the genes involved are located on the toxin plasmid pXO1. Evidence for such a factor was also provided by Cataldi et al. (4, 5). Recently, two classes of *B. anthracis* insertion mutants altered in toxin production were described (15). Members of one class (typified by UM23 tp29 and UM23 tp32) were deficient in production of all three toxin proteins, while the one member of the other class (UM23 tp62) made toxin even in the absence of bicarbonate. Because the three genes coding the toxin proteins are not contiguous on the toxin plasmid, the identification of mutants deficient in the synthesis of all three proteins strongly suggests the existence of a *trans*-acting regulatory gene.

In this report, we describe the cloning of a *trans*-acting positive regulatory gene whose product stimulates *pag* transcription. The DNA sequence of this gene, which we have designated *atxA*, was determined, and it was mapped between *pag* and *cya* on the toxin plasmid. The relationship between *atxA* and the bicarbonate induction of anthrax toxin gene expression is also discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and representative plasmids are listed in Table 1, except that certain widely used *Escherichia coli* strains and cloning vectors are omitted. All strains of *B. anthracis* are derivatives of Weybridge A. Certain plasmids among the pIU series that were

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description[a]	Reference or source
Strains		
B. anthracis		
Weybridge A		
UM23	pXO1$^+$ Tox$^+$ Cap$^-$ Ura$^-$	C. B. Thorne
UM23-1	pXO1$^+$ Tox$^+$ Cap$^-$ Ura$^-$ Str^r; from UM23 by selection for Str^r	C. B. Thorne
UM23C1-1	pXO1$^-$ Tox$^-$ Cap$^-$ Ura$^-$ Str^r; from UM23 by curing of pXO1 and then selection for Str^r	C. B. Thorne
UM23 tp29 and UM23 tp32	pXO1::Tn*917* Tox$^-$ Cap$^-$ Ura$^-$ MLS^r *pag*$^+$ lef$^+$ cya$^+$; from UM23 by Tn*917* mutagenesis	15
UM23 tp62	pXO1::Tn*917* Tox$^+$ Cap$^-$ Ura$^-$ MLS^r; HCO_3-independent toxin synthesis; from UM23 by Tn*917* mutagenesis	15
B. subtilis UOT0277	*his met recE*	32
Plasmids		
pHY300PLK	*E. coli-B. subtilis* shuttle vector; Ap^r Tc^r; replication origin from *Streptococcus faecalis* plasmid pAMα1	15a
pBluescript II SK+ and KS+	Cloning and expression vector; Ap^r	Stratagene
pTK-lac	Vector for integrational mutagenesis of *B. subtilis*; Cm^r Ap^r; ColE1 origin	16
pAT187	Shuttle vector; pAMβ1 origin; Ap^r Km^r	35
pIU4	6.0-kb *Bam*HI fragment of pXO1 containing *pag* promoter and structural gene, in pBluescript II KS+	This study
pTK-PAPlac	1.64-kb *Xba*I-*Hin*dIII fragment from pIU4 containing *pag* promoter, in pTK-lac	This study
pPAPlac	5.1-kb *Eco*RI fragment of pAT187 containing replication origin of pAMβ1, in pTK-PAPlac	This study; see Fig. 2
pIU71	6.0-kb *Bam*HI fragment of pXO1 containing *pag* promoter and structural gene, in pAT187	This study
atxA plasmids		
pIU1	35-kb fragment from partial *Bam*HI digest of pXO1, in pHC79	This study
pIU2	14.2-kb fragment of pXO1, in pHC79; by partial *Hin*dIII digestion and religation of pIU1	This study
pIU10	8.2-kb *Hin*dIII fragment of pXO1, in pHY300PLK; by *Hin*dIII digestion of pIU2	This study
pIU51	5.0-kb *Bgl*II fragment of pIU10, in pHY300PLK; by *Bgl*II digestion of pIU10	This study
pIU52	Same as pIU51 but insert in opposite orientation	This study
pIU55	Same insert as in pIU51, in pBluescript II SK+; by ligation into the *Bam*HI site of pBluescript II SK+	This study
pIU56	Same as pIU55 but insert in opposite orientation	This study
pIU57	2.6-kb *Sna*BI-*Eco*RI fragment of pIU10, in pBluescript II SK+; by deletion of adjacent regions from pIU56	This study
pIU58	2.0-kb fragment of pIU10, extending 3′ from *Sna*BI site, in pBluescript II SK+; by exonuclease III-mung bean nuclease digestion from *Eco*RI site of pIU57	This study
pIU61	4.5-kb *Bam*HI-*Bgl*II fragment of pIU10, in pHY300PLK; by *Bam*HI digestion and religation of pIU51	This study
pIU62	3.0-kb *Sna*BI-*Bgl*II fragment of pIU10, in pHY300PLK; by *Sma*I and *Sna*BI digestion and religation of pIU51	This study
pIU63	2.7-kb *Hpa*I-*Bgl*II fragment of pIU10, in pHY300PLK; by *Sma*I and *Hpa*I digestion and religation of pIU51	This study
pIU64	1.8-kb *Xba*I-*Bgl*II fragment of pIU10, in pHY300PLK; by *Xba*I digestion and religation of pIU52	This study
pIU65	0.4-kb *Eco*RI-*Bgl*II fragment of pIU10, in pHY300PLK; by *Eco*RI digestion and religation of pIU51	This study
pIU66	4.6-kb *Bgl*II-*Eco*RI fragment of pIU10, in pHY300PLK; by *Eco*RI digestion and religation of pIU52	This study
pIU67	4.0-kb *Bgl*II-*Eco*RV fragment of pIU10, in pHY300PLK; by *Sma*I and *Eco*RV digestion and religation of pIU52	This study
pIU68	2.0-kb fragment of pIU10, extending 3′ from *Sna*BI site, in pHY300PLK; by replacement of the 1.8-kb *Xba*I-*Hin*dIII fragment of pIU62 with the 0.7-kb *Xba*I-*Hin*dIII fragment of pIU58	This study

[a] MLS, macrolides-lincosamides-streptogramin B.

generated during cloning of *atxA* are also diagrammed in Fig. 1. Plasmids pIU55, pIU56, pIU57, and pIU58 (not shown in Fig. 1) were constructed principally for DNA sequencing and in vitro transcription-translation. The 5.0-kb *Bgl*II fragment of pIU10, the same fragment present in pIU51 and pIU52 (Fig. 1B), was cloned into pBluescript II SK+ in both orientations to obtain pIU55 and pIU56. Plasmid pIU57 was obtained by deleting from pIU56 the regions 5′ of the *Sna*BI site and 3′ of the *Eco*RI site in the 5.0-kb *Bgl*II *B. anthracis* DNA insert, so that it retains the region shared by pIU62 and pIU66 (Fig. 1B). Plasmid pIU58 was produced from pIU57 by using timed exonuclease III-mung bean nuclease digestion (12) from the *Eco*RI site to remove 638 bp from the end of the insert, after which the end was filled in with the Klenow fragment of DNA polymerase and ligated, thereby removing the region encoding open reading frame 2 (ORF2). The site-specific deletion plasmids pIU61 to pIU67 (Fig. 1) were constructed by digesting pIU51 or pIU52 with appropriate restriction enzymes and subjecting them to self-ligation. To construct pIU68, the 1.8-kb *Xba*I-*Hin*dIII fragment of pIU62 was replaced with the 0.7-kb *Xba*I-*Hin*dIII fragment isolated from pIU58. A plasmid, pIU71, that contains the entire *pag* structural gene in addition to the *pag* promoter and 3.5 kb of upstream DNA was made by treating the 6.0-kb *Bam*HI fragment of pXO1 with the Klenow polymerase to fill in the cohesive ends and ligating this fragment into the *Sma*I site of pAT187 (35).

Media, culture conditions, and measurement of anthrax

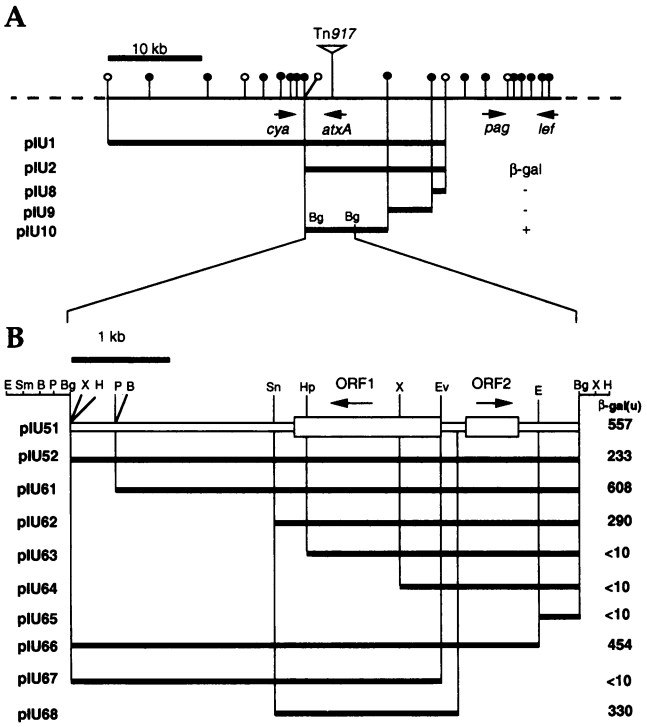

FIG. 1. Cloning and mapping of the *atxA* region. Horizontal bars indicate the DNA fragments retained in the plasmids listed at the left. Symbols indicating restriction sites: ⚲, *Bam*HI; ⚲, *Hin*dIII. Abbreviations for restriction enzymes: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; Hp, *Hpa*I; Sm, *Sma*I; Sn, *Sna*BI; X, *Xba*I. (A) Linear map showing the anthrax toxin gene region on pXO1. Plasmids generated during cloning and localization of the *atxA* region are shown. The approximate positions of the Tn*917* insertions in UM23 tp29 and UM23 tp32 are indicated. Expression of β-galactosidase (β-gal) as indicated by colony color is indicated at the right side as + or −. (B) Deletion analysis of the 5.0-kb *Bgl*II fragment of pIU51 and pIU52 that contains *atxA*. Of the restriction sites shown at the ends of pIU51, only the *Hin*dIII site at the 5′ end and the *Bgl*II site at the 3′ end originated from pXO1; all others were derived from the multiple cloning sites of the vectors used in previous cloning steps. Plasmids pIU52, pIU64, pIU66, and pIU67 have their inserts inverted in the vector relative to the terminal restriction sites shown. β-Galactosidase (β-gal) activities (units per A_{600}) of *B. subtilis*(pPAPlac) transformants containing the deleted plasmids are listed at the right. The two ORFs found by nucleotide sequence analysis are drawn as open bars, and their transcriptional directions are indicated by arrows.

toxin components. *B. anthracis* and *B. subtilis* were grown routinely in LB broth, brain heart infusion (BHI), or R synthetic medium (19) containing 0.02 or 0.05 M Tris HCl and 40 μg of uracil per ml. *B. anthracis* cultures in R medium or BHI were shaken in tightly closed, screw-cap, 250-ml Erlenmeyer flasks. $NaHCO_3$ was added in some cultures to a final concentration of 0.8%. L agar containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Boehringer Mannheim, Indianapolis, Ind.) per ml was used to identify colonies producing β-galactosidase.

PA, LF, and EF in the culture supernatant fluids of *B. anthracis* strains were quantitated by radial diffusion (8) or adenylate cyclase (19) assay. For the experiment shown in Table 2, cultures were shaken at 80 rpm in tightly closed flasks for 17 h at 37°C in R medium containing 0.05 M Tris HCl (pH 8.0) with or without 0.8% $NaHCO_3$. After centrifugation, supernatant fluids were supplemented with 2 mM phenylmethanylsulfonyl fluoride, 5 mM 1,10-*o*-phenanthroline, 4 mM EDTA, and 0.5% horse serum, filtered through 0.22-μm-pore-size membranes, and concentrated 15- or 40-fold in Centriprep30 units (Amicon). Radial immunodiffusion for PA and LF was done in medium containing 1% agarose (SeaKem GTG; FMC BioProducts, Rockland, Maine), 2% polyethylene glycol 8000, 3% fetal bovine serum, 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES; pH 7.5), 0.15 M NaCl, 2 mM EDTA, 0.025% sodium azide, and either 0.5% goat antiserum to PA or 0.7% rabbit antiserum to LF. Standards containing 10 to 50 μg of purified PA or LF per ml were included on each plate. Concentrations of EF were determined in adenylate cyclase assays (19) by comparison with samples of purified EF.

Construction of a *pag-lacZ* transcriptional fusion. First, plasmid pIU4 was constructed by cloning the 6.0-kb *Bam*HI fragment of pXO1 encoding *pag* and 3.5 kb of upstream DNA into pBluescript II KS+. The 1.64-kb *Xba*I-*Hin*dIII fragment of pIU4 was subcloned into pTK-lac, which contains a promoterless *lacZ* gene preceded by a multiple cloning site and the ribosome-binding site of the *B. subtilis spoVG* gene (16). In the resulting transcriptional fusion plasmid, pTK-PAPlac, the *lacZ* gene is under the control of the *pag* promoter. To allow replication in *B. anthracis*, a 5.1-kb *Eco*RI fragment of pAT187 (35) containing the replication origin of pAMβ1 was inserted at the *Eco*RI site, giving pPAPlac (Fig. 2).

DNA methodology and sequencing. Plasmid DNA of *B. anthracis* was isolated as described previously (11). DNA manipulations were carried out by using standard methods (31). DNA was sequenced with a Sequenase kit (United States Biochemical Co., Cleveland, Ohio). Reaction mixtures contained [^{35}S]deoxyadenosine 5′-(α-thio)triphosphate for labeling, and dGTP was replaced by 7-deaza-dGTP to reduce formation of secondary structures. Plasmids derived from pBluescript II were used to generate single-stranded templates for sequencing. *E. coli* XL1-Blue was used as the host strain for pBluescript-derived plasmids. Nested deletions were generated from pIU55 and pIU56 by the method of Henikoff (12) and sequenced by using the United States Biochemical Corp. universal M13 oligonucleotide primer and synthetic oligonucleotides corresponding to sequences within *atxA*. Both strands were sequenced.

The orientations and approximate locations of the Tn*917* insertions in UM23 tp29 and UM23 tp32 that had been previously determined by restriction mapping (14, 15) were determined more precisely by polymerase chain reaction (PCR). Template pXO1 DNA isolated from these strains (11) was amplified with a 20-mer (ACCGTTCTTTCCTGTAC ATC) corresponding to the reverse complement of nucleotides 2479 to 2458 of the sequenced *atxA* DNA and a second 20-mer (TCCCACTAAGCGCTCGGGAC) corresponding to nucleotides 5331 to 5350 in Tn*917*. Amplification cycles were 1 min at 94°C, 2.3 min at 55°C, and 3 min at 72°C for 30 cycles, followed by a final extension for 7 min at 72°C.

DNA sequences were analyzed and data base searching was performed with the Genetics Computer Group programs, MacVector, and the program BLOCKS for detection of distant homologies to families of proteins (13), the latter accessed via an Internet server at the address blocks@howard.fhcrc.org.

In vitro labeling of plasmid-encoded proteins. Proteins encoded by recombinant plasmids were identified by using an *E. coli*-derived in vitro transcription-translation kit (Promega). The [^{35}S]methionine-labeled proteins were sepa-

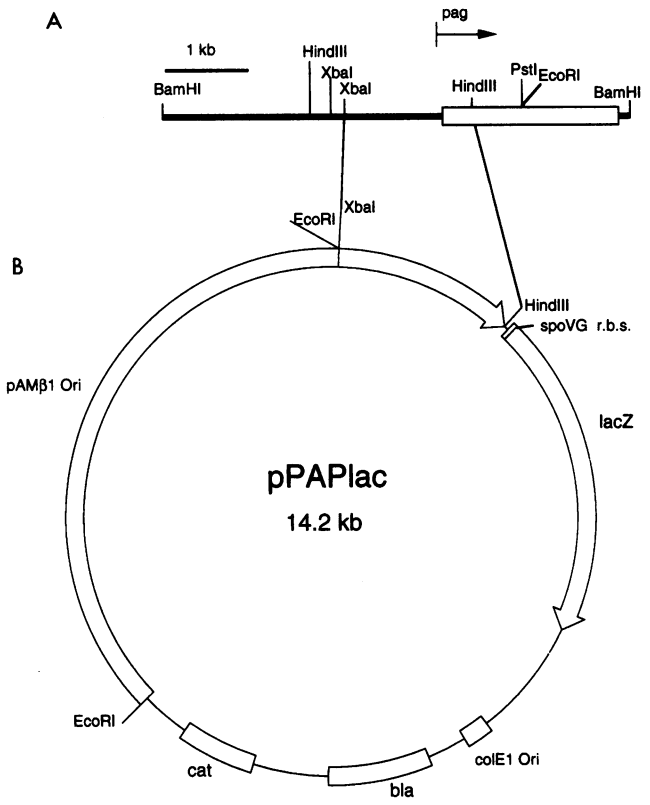

FIG. 2. Construction of a *pag-lacZ* transcriptional fusion. (A) Linear map of the 6.0-kb pXO1 *Bam*HI fragment that contains the entire *pag* gene. The open bar is the *pag* structural gene. The approximate start site and direction of transcription from the *pag* promoter are indicated by the arrow. (B) Map of the *pag-lacZ* transcriptional fusion vector, pPAPlac, constructed by insertion of the *Xba*I-*Hin*dIII fragment of *B. anthracis* DNA into pTK-lac and addition of the 5.1-kb *Eco*RI fragment of pAT187 that contains the replication origin (Ori) of pAMβ1. Directions of *pag* and *lacZ* transcription are indicated by arrows. spoVG r.b.s., ribosome-binding site of the *B. subtilis spoVG* gene, which is positioned to initiate translation of the *lacZ* coding sequence. Other features: bla, β-lactamase gene from pBR322; cat, *cat* gene from pC194; colE1 Ori, replication functions of ColE1.

rated by electrophoresis through a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel. Samples were dissolved in 0.04 M Tris HCl (pH 6.8)–0.05 M dithiothreitol–1% SDS–5% glycerol–0.05 mg of bromophenol blue per ml and heated at 100°C for 5 min prior to electrophoresis. Gels were stained, dried, treated with En^{3}Hance (Dupont), and exposed to X-ray film for fluorography.

β-Galactosidase assay. *B. subtilis* or *B. anthracis* strains carrying pPAPlac were grown in R medium or BHI medium. Samples (1 ml) were collected after cultivation for 16 h. Cells were harvested by centrifugation, washed in ice-cold 50 mM Tris HCl (pH 7.5), and frozen at −20°C. Cells were thawed in Z buffer (25), treated at 37°C with lysozyme (0.5 mg/ml for 5 min for *B. subtilis*; 2.0 mg/ml for 30 min for *B. anthracis*), and lysed by adding Triton X-100 to 0.1%. β-Galactosidase was assayed according to the method of Miller (25), and activity was normalized to the A_{600} of the culture.

Transformation. Transformation of *B. subtilis* UOT0277 (32) cells was performed by the method of Dubnau and Davidoff-Abelson (6). Plasmid DNA for transformation was extracted from the *recA*$^+$ *E. coli* strain MC1061. Transformation of *B. anthracis* was carried out by the method of Quinn and Dancer (28), using DNA extracted from *E. coli* JM110 (*dam dcm*) to obtain unmethylated DNA.

Nucleotide sequence accession number. The nucleotide sequence determined in this study (Fig. 3) is deposited with GenBank under accession number L13841.

RESULTS

Construction of a *pag-lac* transcriptional fusion for measuring *pag* promoter activity. Because the anthrax toxin proteins are susceptible to proteolytic degradation and assays of their activity are not convenient, we constructed a fusion of the *pag* promoter and upstream regions to the gene encoding β-galactosidase and placed this fusion on a plasmid that replicates from the origin of pAMβ1. The resulting plasmid, pPAPlac (Fig. 2B), contains a 1,642-bp *Xba*I-*Hin*dIII fragment of pXO1 that contains 382 bp encoding the PA signal peptide and 98 amino acids of the mature PA protein, as well as 1,260 bp of untranslated upstream DNA. Translation of PA terminates at a stop codon in the multiple cloning site originating from the parental vector pTK-lac (16), and translation then reinitiates at the *lacZ* initiation codon just downstream of the *spoVG* ribosome-binding site. Therefore, the amount of β-galactosidase synthesized reflects the activity of the *pag* promoter. *B. anthracis* strains containing pXO1 and transformed with pPAPlac synthesized β-galactosidase in response to the addition of $NaHCO_3$ (see below), indicating that the reporter plasmid was responding as expected for the *pag* promoter.

Cloning of the *trans*-acting positive regulatory gene (*atxA*). In previous studies, transposon mutagenesis of *B. anthracis* Weybridge A UM23 with the Tn*917*-containing vector pTV1 (43) produced mutants UM23 tp29 and UM23 tp32, which are deficient in production of all three toxin components (14, 15). Restriction mapping showed that Tn*917* had inserted between *cya* and *pag* on plasmid pXO1 in both strains, at the position shown in Fig. 1A, suggesting that this region contains one or more genes required for toxin production (15). We designated the putative regulatory gene *atxA*. The region between *cya* and *pag* contains two *Bam*HI sites which define a 14-kb fragment expected to contain *atxA*. The Tn*917* insertion in UM23 tp62 was mapped several kilobases to the right of the region depicted in Fig. 1A (data not shown).

To clone *atxA*, pXO1 isolated from *B. anthracis* Weybridge A UM23-1 was partially digested with *Bam*HI, and the resulting fragments were ligated to the cosmid vector pHC79; this procedure was followed by in vitro packaging and infection of *E. coli* HB101. One of the 20 clones examined (pIU1) contained a 35-kb insert that yielded the expected 14-kb fragment after *Bam*HI digestion (Fig. 1A). Cosmid pIU1 was partially digested with *Hin*dIII and self-ligated to reduce the insert to the 14.2-kb *Hin*dIII-*Bam*HI fragment that contains the desired 14-kb *Bam*HI fragment (pIU2; Fig. 1A). The 1.0-kb *Hin*dIII-*Bam*HI, 5.0-kb *Hin*dIII, and 8.2-kb *Hin*dIII fragments of pIU2 were ligated into the multiple cloning site of shuttle vector pHY300PLK to produce pIU8, pIU9, and pIU10, respectively. To determine whether these plasmids contained an intact, functional *trans*-acting gene, they were individually transformed into *B. subtilis* containing pPAPlac. Colonies of *B. subtilis* transformants containing pIU10 were blue on agar plates containing X-Gal, whereas those containing pIU8, pIU9, or pPAPlac alone were colorless. These results showed that *atxA* is on the 8.2-kb *Hin*dIII fragment. The 5.0-kb *Bgl*II fragment of

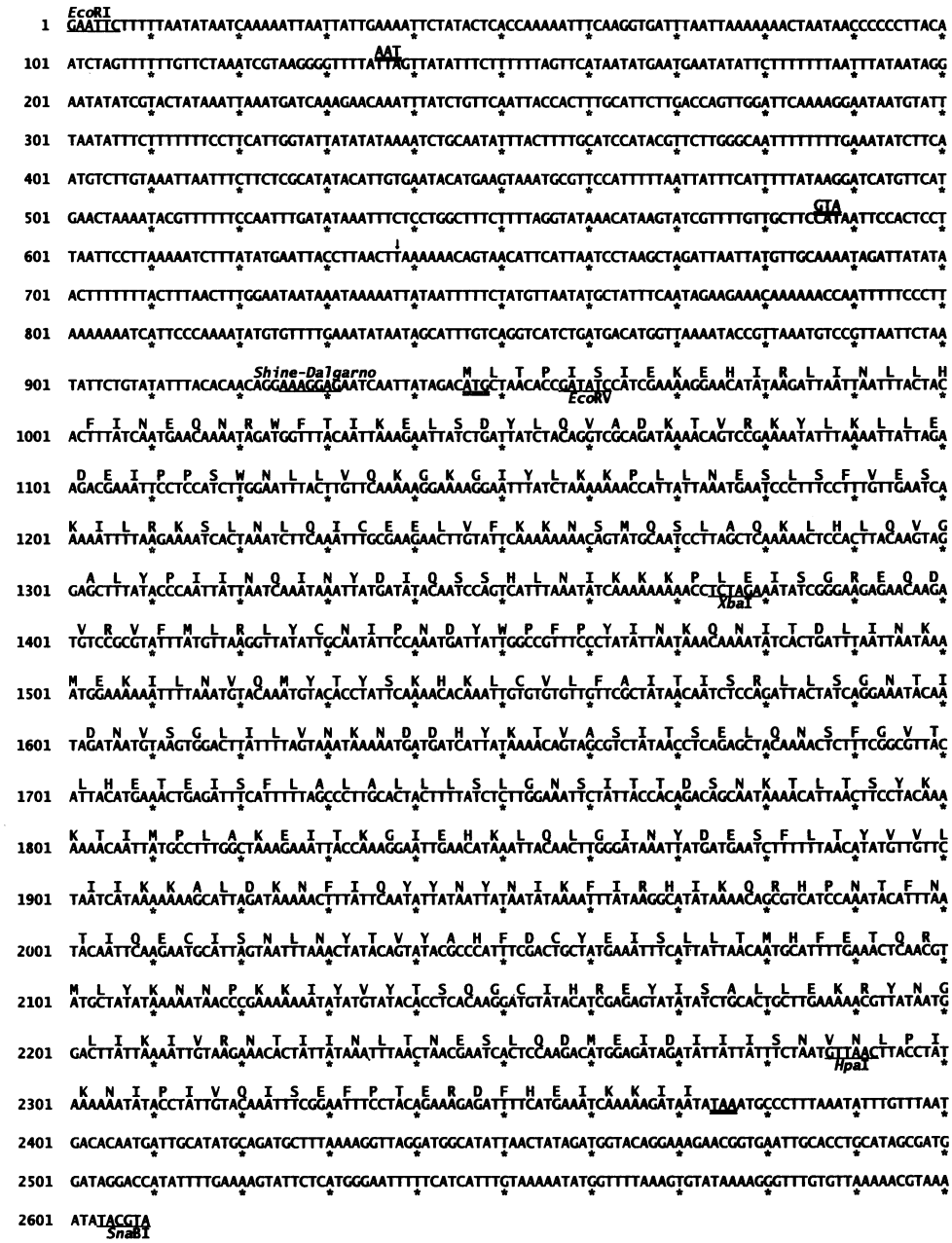

FIG. 3. Nucleotide and deduced amino acid sequences of the *Eco*RI-*Sna*BI fragment of pXO1 containing *atxA*. The sequence shown is of the minus strand as depicted in Fig. 1. ORF1 is nucleotides 946 to 2373, with the initiation and termination codons shown by double underlining. In ORF1, single underlining at nucleotides 925 to 931 identifies the putative Shine-Dalgarno ribosome-binding site. The initiation and termination codons of ORF2, which is encoded by the opposite strand, are shown by double underlining at nucleotides 588 and 139, respectively. The endpoint of the deletion in pIU68 at nucleotide 638 is indicated by a vertical arrow.

pIU10 was then inserted in the *Bgl*II site of pHY300PLK, yielding pIU51 and pIU52, which contain the same fragment in opposite orientations (Fig. 1B). To precisely locate *atxA* within this fragment, site-specific deletion mutants were constructed from pIU51 and pIU52. *B. subtilis* containing pPAPlac was transformed with these deletion mutants, and the transcriptional activity of the *pag* promoter was measured by assaying β-galactosidase. High levels of β-galactosidase were found in transformants containing pIU51, pIU52, pIU61, pIU62, and pIU66, whereas only background levels were found with pIU63, pIU64, pIU65, and pIU67 (Fig. 1B). These results showed that *atxA* is located entirely within the 2.6-kb *Sna*BI-*Eco*RI fragment. This fragment was subcloned to yield pIU57.

DNA sequence determination of the *atxA* locus. The nucleotide sequence of the 2,609 bp of the *Sna*BI-*Eco*RI fragment in pIU57 was determined (Fig. 3). Two large ORFs with opposite orientations were found (ORF1, 1,428 nucleotides; ORF2, 450 nucleotides). ORF1 and ORF2 are predicted to encode proteins of 476 and 150 amino acids with molecular

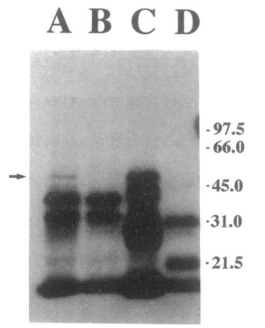

FIG. 4. In vitro transcription-translation of *atxA* DNA in an *E. coli* extract. Plasmids pIU62 and pIU57, both of which contain ORF1 (*atxA*) and ORF2, were used as templates for in vitro transcription and translation. The [^{35}S]methionine-labeled products were electrophoresed through an SDS–10% polyacrylamide gel, which was dried and exposed by fluorography. Templates for the reactions: lane A, pIU62; lane B, pHY300PLK (vector control for pIU62); lane C, pIU57; lane D, pBluescript II SK+ (vector control for pIU57). The arrow at the left indicates the *atxA* product. Sizes are indicated in kilodaltons.

weights of 55,673 and 18,227, respectively. The probable Shine-Dalgarno ribosome-binding site for translation of ORF1, AAAGGAG, is separated from the ATG initiation codon by 14 nucleotides. In ORF2, the putative ribosome-binding site, AAGGAG, is separated by only 7 nucleotides from the probable translational start codon. Sequencing located the *Eco*RV site that defines the 3′ end of the insert in pIU67 (Fig. 1B) as being 8 nucleotides downstream of the ATG initiation codon (Fig. 3), showing that ORF1 is disrupted in this plasmid. The DNA sequence was used to search the GenBank and EMBL data bases, and the deduced amino acid sequences of ORF1 and ORF2 were used to search the PIR and Swiss Protein data bases. No significant similarities to known genes were detected.

Evidence that ORF1 is involved in regulation was obtained by PCR mapping of the Tn*917* insertion point in mutants UM23 tp29 and UM23 tp32. PCR using one primer from within the *atxA* sequence and another from the Tn*917* sequence produced fragments of about 500 bp from both UM23 tp29 and UM23 tp32 (data not shown), indicating that Tn*917* was inserted near nucleotide 1800 of the sequenced DNA (Fig. 3), approximately in the middle of ORF1.

Involvement of ORF1 in activation of the *pag* promoter; characterization of the protein products of the ORFs. The deletion analysis shown in Fig. 1B and the demonstrations that ORF1 was interrupted in UM23 tp29 and UM23 tp32 and incomplete in pIU67 all indicated that ORF1 is required for activation of *pag* transcription. However, these data did not test whether the product of ORF2 is also required. To resolve this question, pIU62 was further shortened to produce pIU68, which contains ORF1 but not ORF2. As shown in Fig. 1B, *B. subtilis*(pPAPlac) transformed with pIU68 produced almost the same amount of β-galactosidase as did the transformant with pIU62, indicating that ORF2 is not required for *trans*-activation of *pag* transcription. These data establish that ORF1 alone constitutes the positive regulatory gene. Henceforth, the designation *atxA* will refer to ORF1.

An in vitro transcription-translation system was used to examine the protein products produced from plasmids pIU62 and pIU57, both of which contain ORF1 (*atxA*) and ORF2 (Fig. 4). In addition to products encoded by the corresponding vectors pHY300PLK and pBluescript II SK+, a product of about 53 kDa was observed, consistent with the size calculated for ORF1 (*atxA*). The reaction using pIU57 produced more of the 53-kDa product, along with smaller polypeptides that are probably proteolytic fragments of the 53-kDa protein. Furthermore, an 18-kDa protein having the size expected for the ORF2 product was also observed on an SDS–12.5% polyacrylamide gel (data not shown).

Stimulation by *atxA* of PA production from a cloned *pag* gene. The data presented above showed that *atxA* acts to enhance transcription from the *pag* promoter when provided in *trans*. To examine whether *atxA* stimulates PA production from the original *pag* gene region, we inserted the 6.0-kb *Bam*HI *pag* fragment from pXO1 into the shuttle vector pAT187 to produce pIU71 and then transformed this plasmid into *B. anthracis* UM23C1-1, which is cured of pXO1. Production of PA was not observed in the resulting UM23C1-1(pIU71) strain but was obtained when the *atxA* gene was subsequently introduced into this strain by transformation with pIU68 (Table 2). In this case, PA expression was obtained in both the absence and presence of $NaHCO_3$.

Complementation of insertion mutants with the cloned *atxA*. The existence of a positive regulatory gene was initially recognized by demonstrating that Tn*917* insertion mutants UM23 tp29 and UM23 tp32 failed to produce detectable amounts of the three toxin proteins. To determine whether the cloned *atxA* gene would complement these mutations, the mutant strains were transformed with pIU68, the construct containing only *atxA*. Concentrations of all three toxin components were measured in the culture filtrates of the wild-type strains and the transformants (Table 2). Controls included (i) the parental UM23-1 containing a wild-type pXO1 plasmid and (ii) strains transformed with pHY300PLK, the parent vector of pIU68. Consistent with prior results, the insertion mutants made only negligible amounts of PA, LF, and EF, even when 0.8% $NaHCO_3$ was added to the R medium. Introduction of pIU68 into each strain restored the ability of the mutants to produce the three toxin components in the presence of $NaHCO_3$. Thus, the *atxA* gene encodes a *trans*-acting product which stimulates expression of not only *pag* but also *cya* and *lef*. The production of the three toxin components by the complemented strains was stimulated by addition of $NaHCO_3$ to the medium, paralleling the phenotype of parental strain UM23-1. Assays of toxin production in the Tn*917* mutants complemented with pIU68 were repeated several times, and although absolute amounts of toxin differed somewhat, the responses to the presence of pIU68 and to addition of $NaHCO_3$ were essentially like those shown in the Table 2.

We also included UM23 tp62 in the experiment shown in Table 2. This mutant strain produces all three toxin components even in the absence of $NaHCO_3$, presumably as a result of inactivation of a negative regulatory gene. Introduction of the *atxA* gene on pIU68 caused no additional increase in toxin yield in the presence of $NaHCO_3$ but appeared to partially restore the dependence on $NaHCO_3$ for synthesis of toxin.

Activity of the *pag-lacZ* transcriptional fusion in *B. anthracis*. To examine the possible relationship between *atxA* and induction of *pag* transcription by $NaHCO_3$, we measured β-galactosidase activity in extracts of various *B. anthracis* strains containing pPAPlac after growth in the synthetic R medium and in BHI broth (Table 3). The strain cured of pXO1, UM23C1-1, did not produce β-galactosidase under any condition, as expected when *atxA* is absent. For parental strain UM23-1, the increase in enzyme activity caused by addition of $NaHCO_3$ to R medium was 31-fold, while the

TABLE 2. Production of anthrax toxin components by insertion mutants complemented with the cloned *atxA* gene

Strain	Plasmid(s)	Toxin component in supernatant[a] (μg/ml)					
		PA		LF		EF	
		−	+	−	+	−	+
UM23C1-1	pIU71	<0.5	<0.5	ND	ND	ND	ND
	pIU71, pHY300PLK	<0.5	<0.5	ND	ND	ND	ND
	pIU71, pIU68	1.3	1.8	ND	ND	ND	ND
UM23-1	pXO1	<0.5	3.4	<0.25	1.6	<0.05	0.4
	pXO1, pHY300PLK	<0.5	5.0	<0.25	1.5	<0.05	0.3
	pXO1, pIU68	2.9	5.9	1.9	4.4	<0.05	1.4
UM23 tp29	pXO1::Tn*917*	<0.5	<0.5	<0.25	<0.25	<0.05	<0.05
	pXO1::Tn*917*, pHY300PLK	<0.5	<0.5	<0.25	<0.25	<0.05	<0.05
	pXO1::Tn*917*, pIU68	<0.5	2.9	0.9	4.7	<0.05	1.4
UM23 tp32	pXO1::Tn*917*	<0.5	<0.5	<0.25	<0.25	<0.05	<0.05
	pXO1::Tn*917*, pHY300PLK	<0.5	<0.5	<0.25	<0.25	<0.05	<0.05
	pXO1::Tn*917*, pIU68	1.7	4.7	1.3	7.9	0.2	1.2
UM23 tp62	pXO1::Tn*917*	7.8	6.9	1.8	5.5	0.6	2.4
	pXO1::Tn*917*, pHY300PLK	3.3	10.6	1.8	4.8	0.4	2.5
	pXO1::Tn*917*, pIU68	1.8	7.8	0.6	4.2	<0.05	3.9

[a] Concentrations of toxin components in filtrates of 17-h R medium cultures were determined by radial diffusion (PA and LF) or adenylate cyclase assays (EF). − and + indicate absence and presence of 0.8% $NaHCO_3$ during growth. ND, not determined.

increase in BHI medium was only about 5-fold. Mutant UM23 tp32, shown above to have Tn*917* inserted into *atxA*, produced no measurable β-galactosidase under any condition. For mutant UM23 tp62, β-galactosidase activity was also quite high in the absence of $NaHCO_3$, and its addition caused increases of only 2.7- and 4-fold in R medium and BHI medium, respectively. This result is consistent with the previous interpretation (15) that a negative regulator of toxin production is inactivated in UM23 tp62. When strain UM23C1-1 was transformed with pIU68, β-galactosidase activity was very high even in the absence of $NaHCO_3$, and no significant increase was observed upon addition of $NaHCO_3$ to either growth medium.

DISCUSSION

Many bacterial pathogens have developed genetic mechanisms to regulate biosynthesis of their virulence factors (24). Selective pressures will favor mechanisms which limit the energetically expensive synthesis of virulence factors to periods during which the pathogen is growing in host tissues. *B. anthracis* appears to be an example of such a pathogen, because synthesis of the three-component anthrax toxin and of the poly-D-glutamate capsule requires the presence of bicarbonate, a metabolite present in living tissues. The genes for the toxin and the capsule are located on separate plasmids, pXO1 and pXO2, respectively. Each plasmid appears to have genes encoding a bicarbonate-sensing mechanism, because strains cured of either plasmid still show bicarbonate-dependent regulation of the virulence factor encoded by the remaining plasmid (1, 21).

The dependence of *B. anthracis* on bicarbonate was recognized very early in the studies of this pathogen (9, 33). During extensive studies of optimization of anthrax vaccine production, more than 1,000 different medium recipes were examined (42), and it was shown that toxin synthesis was enhanced by certain amino acid mixtures. However, the largest and most consistent stimulation of toxin synthesis is that caused by bicarbonate (41). In our previous report, it was shown that bicarbonate controls *pag* expression by acting at the transcriptional level and that some of the genes responsible for transcriptional activation are located on pXO1 (1). Similar conclusions were drawn by Cataldi et al. (5) by returning a plasmid equivalent to pIU71 to a strain in which *pag* had been inactivated. More recently, these workers have used a *pag-lacZ* transcriptional fusion like that in pPAPlac to confirm that bicarbonate stimulates transcription from the *pag* promoter (4).

In the work described here, we sought to identify and characterize putative genes that regulate toxin biosynthesis. We began by mutagenizing *B. anthracis* UM23 with Tn*917* (14), and we obtained two classes of mutants altered in toxin synthesis (15). The first class, typified by UM23 tp29 and UM23 tp32, was deficient in synthesis of all three toxin

TABLE 3. β-Galactosidase synthesis from *pag-lacZ* transcriptional fusion vector pPAPlac in various *B. anthracis* strains[a]

Strain	Plasmid(s)	β-Galactosidase activity[a] (U)			
		R medium		BHI medium	
		$-NaHCO_3$	$+NaHCO_3$	$-NaHCO_3$	$+NaHCO_3$
UM23C1-1	pPAPlac	<5	<5	<5	<5
UM23-1	pXO1, pPAPlac	19	591	36	173
UM23 tp32	pXO1::Tn*917*, pPAPlac	<5	<5	<5	<5
UM23 tp62	pXO1::Tn*917*, pPAPlac	287	774	194	781
UM23C1-1	pIU68, pPAPlac	713	789	508	493

[a] Strains were grown in the indicated medium with or without 0.8% $NaHCO_3$. Activity was determined by the method of Miller (25). Values are averages of three independent experiments.

proteins. Because the three toxin genes are not located in a single operon on pXO1, these insertion mutations implied the existence of a *trans*-acting positive regulatory gene. The other type of mutant, of which UM23 tp62 is the only representative, produces all three toxin proteins even in the absence of bicarbonate. The ability to act on multiple distant genes argues for the existence of a separate *trans*-acting negative regulator.

The *trans*-acting positive regulatory gene was cloned by using a transcriptional fusion of the *pag* promoter to the β-galactosidase gene as a reporter of transcriptional activity. Restriction mapping had shown that Tn*917* was inserted between *pag* and *cya* in UM23 tp29 and UM23 tp32 (15). A restriction fragment from this region complemented the mutation, and deletion analysis localized the gene to a small region which was then sequenced. Two ORFs were identified, and further deletion analysis and complementation tests showed that the larger one, ORF1, is sufficient to activate *pag* transcription. PCR mapping also showed that the Tn*917* insertions in mutants UM23 tp29 and UM23 tp32 were located in ORF1. We designated the activator gene encoding ORF1 as *atxA*.

The cloned *atxA* determinant encodes a potential polypeptide of 476 amino acids with a molecular weight of 55,673, a value consistent with results obtained with an in vitro transcription-translation system (Fig. 4). The probable Shine-Dalgarno ribosome-binding site of *atxA* at nucleotide 935, AAAGGAG, is identical to those of *pag* (40), *lef* (2), and *cya* (7, 30). The spacing between this ribosome-binding site sequence and the *atxA* initiation codon, 14 nucleotides, is greater than the 7- to 9-nucleotide spacers found in the toxin genes. *B. subtilis* is quite tolerant of long spacers, as indicated by the finding that a 13-nucleotide spacer gives a translational efficiency in a model system that is 50% of maximum (39). If the translational mechanism in *B. anthracis* is like that in *B. subtilis*, as seems probable, the putative ribosome-binding site of *atxA* can be expected to be functional. ORF2 also has an acceptable ribosome-binding site, AAGGAG, with a spacer of 7 nucleotides, equal to that of *pag* and at the lower limit of efficient spacers (39).

The regions immediately 5′ of *atxA* do not contain sequences that can be identified as promoters or transcriptional start sites. Thus, there are no sequences that match the −10 and −35 sequences recognized by any of the nine sigma factors characterized in *B. subtilis* (27). Mapping of the transcriptional start site may help to locate the promoter and explain how and whether *atxA* transcription is regulated. We anticipated that obtaining the sequence of the *atxA* gene would identify it as a member of a previously recognized family of transcriptional regulator genes. However, extensive efforts to find proteins in the data bases with statistically significant sequence similarities to ORF1 (*atxA*) and ORF2 were unsuccessful.

We have reexamined the sequences upstream of the *pag*, *lef*, and *cya* structural genes to find regions that might be involved in controlling transcription. As noted previously (40), upstream of *pag* there are exact (TATAAT) and nearly exact (TTGAAA versus TTGACA) matches to the −10 and −35 consensus sequences recognized by the primary housekeeping σ^A of *B. subtilis* RNA polymerase (27). The implication that *pag* is transcribed during exponential growth phase is consistent with a prior demonstration that the amount of lethal toxin parallels cell density (8) and with a recent report that expression from the *pag* promoter occurs throughout the exponential growth phase (4). Another notable feature of the putative *pag* promoter region is that the −10 and −35 sequences are potentially sequestered within an extended stem-loop structure, nucleotides 1722 to 1779 (40), having a ΔGf of −15.8 kcal (ca. −66.1 kJ)/mol. In regard to transcriptional regulation of *lef*, *cya*, and *atxA*, no sequences matching the consensus −10 and −35 promoter sequences recognized by σ^A have been located.

The *B. subtilis* strains containing the *pag-lacZ* fusion and the different *atxA* plasmids appeared to differ in β-galactosidase production (Fig. 1), with values varying from 200 to 600 U. However, these differences are not considered statistically significant, and they should not be used to infer that regions adjacent to ORF1 (*atxA*) contribute to *pag* regulation. The ability of plasmids containing only *atxA* (e.g., pIU68) to strongly activate transcription of the *pag-lacZ* fusion in *B. subtilis* shows that this gene is the most important activator of *pag* transcription. It remains possible that additional genes that modulate transcription of the toxin genes will later be found adjacent to *atxA* or located elsewhere.

The complementation of the mutations in UM23 tp29 and UM23 tp32 by cloned *atxA* (Table 2) showed that the phenotypes of these mutants were the result of single Tn*917* insertions and further supported the view that the *atxA* product acts in *trans* on the promoters of *pag*, *lef*, and *cya*. It was interesting to note that the strains complemented by pIU68, particularly UM23-1 and UM23 tp32, produced some PA, LF, and EF in the absence of bicarbonate, in contrast to the parent (uncomplemented) strain, UM23-1, which showed an absolute requirement for bicarbonate (Table 2). These differences may reflect gene copy number effects. The 184-kb plasmid pXO1 is expected to have a very low copy number, whereas the pHY300PLK-based vector pIU68 may have a copy number of 5 to 20. Thus, in the complemented strains, the *atxA* product is likely to be produced from pIU68 at a higher ratio to the *pag* target DNA sequences than in the case in which both *atxA* and the *pag* target DNA are on pXO1. The situation is complicated by the fact that the putative negative regulatory gene on pXO1 is intact in UM23 tp29 and UM23 tp32, so that a normal level of the putative negative regulatory product is present in these strains. The partial alleviation of the bicarbonate requirement in UM23 tp32(pIU68) could result from an excess of the *atxA* product successfully competing with the low, normal level of the negative regulator. This interpretation is consistent with the data obtained when the *pag* promoter and structural gene were supplied on the low-copy-number plasmid pIU71 and tested in a strain cured of pXO1 and therefore lacking the negative regulatory gene (UM23C1-1; Table 2). In this case, the addition of $NaHCO_3$ had no significant effect, as might be expected if the *atxA* product was in excess over *pag* target DNA sequences and no negative regulator was present to compete.

To further characterize the interactions between *atxA*, the *pag* promoter, and the putative negative regulatory gene, the activity of the *pag-lacZ* fusion was measured in several *B. anthracis* strains (Table 3). Two different media were compared because previous work showed that the induction of PA synthesis by bicarbonate is medium dependent (1). Although β-galactosidase synthesis was higher in most cases in R medium, qualitatively similar responses to $NaHCO_3$ were found in BHI medium cultures. In a genetic background containing the functional regulatory genes supplied in low copy number by pXO1, enzyme synthesis was highly dependent on bicarbonate. In strain UM23 tp62, substantial levels of β-galactosidase were produced in the absence of bicarbonate, consistent with the ability of this strain to make

all three toxin proteins in the absence of bicarbonate and with the interpretation that a negative regulatory gene has been inactivated in this mutant. A small additional increase was induced by bicarbonate, suggesting that the negative regulator may not be completely inactivated by the mutation in UM23 tp62. The highest β-galactosidase synthesis was seen in the strain containing *atxA* on a multicopy plasmid and lacking the negative regulatory gene (i.e., cured of pXO1). In this case, bicarbonate had no effect, as might be predicted if its normal action is to inhibit the action of the negative regulatory gene product.

The data presented here show that *B. anthracis* contains a positive regulatory gene whose product acts in *trans* to activate toxin synthesis. Additional evidence is presented describing a negative regulatory gene involved with the induction of toxin synthesis by bicarbonate. The data are consistent with a model in which the negative regulatory gene encodes a repressor which binds to DNA in the absence of bicarbonate. The repressor could block transcription of either *atxA* or the toxin genes. There are many precedents for control of transcription by small molecules such as simple sugars, but in few of these cases are inorganic ions the active agents. The two other cases in which bicarbonate has been implicated as an environmental signal are the enhancement of polysaccharide capsule synthesis in *Cryptococcus neoformans* (10) and the evidence for induction of M-protein transcription in *Streptococcus pyogenes* (3). Cloning and sequencing of the *B. anthracis* negative regulatory gene are under way, with the expectation that these will help to clarify the regulatory networks in this pathogen and perhaps contribute insights applicable to other microorganisms.

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